Failure to Detect Human T-Lymphotropic Virus Type-I Proviral DNA in Cell Lines and Tissues from Patients with Cutaneous T-Cell Lymphoma

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Previous molecular studies investigating the presence of HTLV-I proviral DNA in cell lines and tissue samples of patients with cutaneous T-cell lymphoma (CTCL) have reported a detection rate ranging from 0–92%. Despite the lack of epidemiologic data linking HTLV-I infection with CTCL, the molecular data still invite speculation regarding the precise role of HTLV-I in the pathogenesis of CTCL. To determine the detection rate of HTLV-I proviral DNA among CTCL patients referred to our medical center, we analyzed Epstein–Barr virus–transformed cell lines established from peripheral blood of seven CTCL patients and 43 tissue samples from 22 patients with different stages of disease. Genomic DNA was polymerase chain reaction-amplified with primers within the HTLV-I *tax* gene region. Amplification products were probed with nested oligonucleotide probes by Southern blot analysis. No HTLV-I proviral sequences were detected in the samples (0/50). Using HTLV-I/II *pol* primers, no HTLV-I *pol* gene sequences were detected. In tissues from one patient, HTLV-II *pol* and *tax* gene sequences were detected; however, HTLV-II proviral integration was not detected by Southern blot analysis of the genomic DNA. Our data suggest: (i) HTLV-I does not appear to be a primary etiologic agent in CTCL; and (ii) HTLV-II *pol* and *tax* gene sequences can be detected in a minority of CTCL patients, but this does not necessarily imply an etiologic role. ***Key words: mycosis fungoides/Sezary syndrome/PCR amplification. J Invest Dermatol 107:308–313, 1996***

Cutaneous T-cell lymphoma (CTCL), a malignancy of mature T lymphocytes (T cells), appears first in the skin and slowly progresses through stages of increasing cutaneous and extracutaneous involvement (Greer *et al*, 1993). CTCL has two clinical variants: (i) mycosis fungoides, which classically evolves slowly through progressive stages of cutaneous involvement (patch, plaque, tumor and/or erythroderma) with progression to extracutaneous involvement (lymph nodes and viscera) and (ii) Sezary syndrome, a leukemic variant, which presents with generalized exfoliative erythroderma, lymphadenopathy, and leukocytosis with atypical circulating lymphocytes with hyperconvoluted nuclear contours (Sezary cells) (Lutzner *et al*, 1971).

The etiology of CTCL is unknown, but a retroviral etiology has been an attractive hypothesis (MacKie, 1981) since the discovery of the first human retrovirus, HTLV-I, and its association with adult T-cell leukemia/lymphoma (ATL) (Poiesz *et al*, 1980). The most intriguing support for an association of HTLV-I and CTCL has been from a series of reports demonstrating evidence of retroviral DNA in cell lines and tissues of patients with CTCL (Manzari *et al*, 1987; Anagnostopoulos *et al*, 1990; Detmar *et al*, 1991; Hall *et al*, 1991; Zucker-Franklin *et al*, 1991; D'Incan *et al*, 1992; Srivastava *et al*, 1992; Zucker-Franklin *et al*, 1992; Bazarbachi *et al*, 1993; Chan *et al*, 1993; Whittaker and Luzzatto, 1993; Ghosh *et al*, 1994; Manca *et al*, 1994; Pancake *et al*, 1995).

Manzari *et al* (1987) reported the molecular detection of retroviral DNA in a single cell line established from the peripheral blood of a patient with Sezary syndrome and concluded that they had isolated a new retrovirus (designated HTLV-V), which was closely related to HTLV-I and associated with CTCL. Unfortunately, there has been no additional confirmatory data since the time of the initial report to corroborate the existence of HTLV-V. Hall *et al* (1991) reported the detection of a defective or truncated HTLV-I provirus in an Epstein–Barr virus (EBV)-transformed B-cell line developed from peripheral blood mononuclear cells from a Sezary syndrome patient, providing evidence that defective forms of HTLV-I may play a role in the development of CTCL. Zucker-Franklin *et al* generated cell lines from peripheral blood mononuclear cells from patients with early stages of mycosis fungoides and detected HTLV-I DNA sequences in up to five of nine cell lines by polymerase chain reaction (PCR) amplification (Zucker-Franklin *et al*, 1991, 1992). Subsequently, HTLV-I/II proviral sequences have been detected by PCR amplification in variable percentages in the CTCL tissues, with reports ranging from 0% (Capesius *et al*, 1991; Lisby *et al*, 1992; Bazarbachi *et al*, 1993; Boni *et al*, 1996) up to 92% (Pancake *et al*, 1995).

Based on these preliminary observations, we sought to confirm and extend these findings by determining whether HTLV-I could

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Abbreviations: ATL, adult T-cell leukemia/lymphoma; CTCL, cutaneous T-cell lymphoma; EBV, Epstein–Barr virus; HTLV, human T-lymphotropic virus.

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be detected in EBV-transformed cell lines and cutaneous lesions at different disease stages as well as in a wide range of extracutaneous sites in patients with CTCL.

MATERIALS AND METHODS

Patients Tissue samples were obtained from 22 patients after informed consent was obtained. Seventeen patients had clinical, histopathologic, and immunohistologic findings diagnostic of CTCL (Edelson, 1980; Murphy, 1988; Vowels *et al*, 1994). One patient had CTCL and concurrent Hodgkin's disease; the Hodgkin's disease diagnostic lymph node biopsy is included in our analysis. Four patients had clinical, histopathologic, and immunohistologic findings diagnostic of parapsoriasis, a "precursor state" of CTCL (Edelson, 1980). Two of these four patients went on to develop unequivocal CTCL. The clinical stage (Bunn and Lamberg, 1979) of each patient is listed in **Table I**.

Cell Lines EBV-transformed cell lines were established from the peripheral blood of seven patients with erythrodermic CTCL (Sezary syndrome) and three healthy adult controls as previously described (Hudson and Hays, 1989). Briefly, peripheral blood mononuclear cells were prepared from heparinized whole blood by isolation on a ficoll gradient. Approximately $5–10 \times 10^6$ peripheral blood mononuclear cells were resuspended in 200 μl of heat-inactivated fetal bovine serum (FBS) to which were added 2 ml of cell-free supernatant collected from B95-8 cells and 10 ng polybrene per ml. The cells were incubated for 2–4 h at 37°C in 5% CO_2. Following two washes with phosphate buffered saline, the cells were resuspended at a concentration of 1.5×10^6 cells per ml in RPMI 1640 medium supplemented with 20% FBS and 0.5 μg cyclosporin per ml, and 2-ml aliquots were plated in a 24-well culture plate. Each week, 1 ml of supernatant was removed and replaced with fresh medium without cyclosporin. After 3–4 wk, visible clumps of cells were apparent, and the culture was transferred to a 25-cm^2 flask for expansion. The cells were maintained at a density of 10^6 per ml in RPMI 1640 and 10% FBS. All cell culture reagents were obtained from GIBCO BRL (Gaithersburg, MD). Mycoplasma testing of culture supernatant by standard indirect DNA fluorescence assay failed to demonstrate evidence of mycoplasma contamination. Histochemical analysis was performed on cytospin preparations with CD19 (B cell) and CD2 (T cell) monoclonal antibodies (Becton Dickinson, San Jose, CA) (Finn *et al*, 1996).

DNA Extraction Snap-frozen specimens were processed by standard techniques of cell lysis, proteinase-K digestion, phenol extraction, and ethanol precipitation (Lessin *et al*, 1991). Dedicated laboratory space, equipment, reagents, and supplies were utilized to control for contamination. All DNA extraction was performed in laboratory space in which no known previous retroviral experimentation occurred.

Southern Blot Analysis Restriction enzyme digestion, electrophoresis, blotting and ultraviolet cross-linking of nylon filters (Zetabind, Cuno, Inc., Meriden, CT), hybridization, washing of filters, autoradiography, and nick translation of radiolabeled probes were performed as previously described (Lessin *et al*, 1988). Probes for immunoglobulin heavy chain gene (Gauwerky *et al*, 1988), T-cell receptor β locus (Russo *et al*, 1988), EBV (gift from Dr. G. Rovera), HTLV-I (Reddy *et al*, 1988), and HTLV-II (ATCC, Rockville, MD) were used.

PCR Amplification A commercially available deoxyuracil triphosphate (dUTP) substitution kit (GIBCO BRL) was utilized in all PCR reactions to control for carry-over contamination. Following manufacturer's recommendations, 0.5–1.0 μg of genomic DNA was mixed in a total volume of 50 μl within standard buffer, 1.5 mM $MgCl_2$, 1.25 mM deoxyuracil-deoxynucleotide triphosphates mix, 100 ng (15 pmol) of 3′ and 5′ primers (see below), 2.5 units of *Taq* polymerase, and 1 unit of uracil DNA glycoylase. Reaction mixtures were incubated at 37°C for 10 min, then heated at 94°C for 8 min before 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Oligonucleotide primers for conserved sequences of the *pol* regions for HTLV-I/II (3′-SK110; 5′-SK111) (Kwok *et al*, 1988) (Research Genetics, Huntsville, AL) were used for HTLV-I/II *pol* gene detection, whereas two sets of primers, pX-5′ and pX-3′ (Hall *et al*, 1991) or SK43 and SK44 (Kwok *et al*, 1988) (Research Genetics), were used for amplification of HTLV-I or HTLV-I/II *tax* gene sequences, respectively. Amplification products were sized on 2% agarose gels, blotted, and probed with ^{32}P-labeled nested probes (Lessin *et al*, 1991) for HTLV-I (pX-N) (Hall *et al*, 1991) or HTLV-I/II *tax* (SK45) or HTLV-I *pol* (SK112) and HTLV-II *pol* (SK188) (Kwok *et al*, 1988) (Research Genetics). Integrity of all DNA samples was verified by PCR with primers detecting β-actin gene (Research Genetics) and/or T-cell receptor-γ gene rearrangement (Bourguin *et al*, 1990). DNA from an HTLV-I–infected cell line (MT-2) and an HTLV-II infected cell line (MoT) served as positive controls (gifts from Drs. R. Mettus and J. Hoxie). PCR was performed in areas that were dedicated to such procedures and that were free of plasmid and phage work. In addition, we used reagents, water, supplies, and equipment dedicated only to PCR. Amplification and nonamplified samples were handled separately. All PCR amplification with dUTP and Southern blot work were performed in the Department of Dermatolgy Laboratories on the second floor of the Clinical Research Building of the University of Pennsylvania. Prior to these experiments, no work with retroviruses and no PCR amplification of HTLV occurred in this facility. The dUTP substitution kit was used in the very first PCR performed with HTLV primers in this facility and has been used ever since. As a result, we have tightly controlled for contamination from PCR product carry-over.

Table I. Summary of Patient Samples: No Detection of HTLV-I

Patient	Diagnosis[a]	Stage[b]	Tissue[c] (status[d])	PCR / Southern Blot pX I[e]	pol I[e]	pol II[e]
1.	PP	0	PCH (−)	−	−	−
2.	PP	0	PCH (−)	−	−	−
3.	PP	0	PCH (−)	−	−	−
4.	PP	0	PCH (−)	−	−	−
5.	MF	Ia	PCH (+)	−	−	−
6.	MF	Ia	PCH (+)	−	−	−
7.	MF	Ia	PCH (+)	−	−	−
8.	MF	Ia	PCH (+)	−	−	−
9.	MF	Ia	PCH (+)	−	−	−
			PBL (−)	−	−	−
10.	MF	Ia	PCH (+)	−	−	−
			PBL (−)	−	−	−
11.	MF	Ib	PLQ (+)	−	−	−
			TMR (+)	−	−	−
12.	MF	IIa	LN (−)	−	−	−
13.	MF	IIb	PLQ (+)	−	−	−
			TMR (+)	−	−	−
14.	MF	IIb	TMR (+)	−	−	−
15.	MF	IIb	PLQ (+)	−	−	−
			TMR (+)	−	−	−
16.	SS	III	PBL-1991 (−)	−	−	−
			PBL-1992 (+)	−	−	−
17.	MF	IV	PLQ (+)	−	−	−
			PBL (+)	−	−	−
			LN (+)	−	−	−
18.	MF	IV	TMR (+)	−	−	−
			PBL (+)	−	−	−
			BM (−)	−	−	−
			LN (+LCT)	−	−	−
19.	SS	IV	PLQ (+)	−	−	−
			PBL (+)	−	−	−
			BM (+)	−	−	−
			LN (+LCT)	−	−	−
20.	SS	IV	TMR (+)	−	−	−
			TMR (+LCT)	−	−	−
			PBL (+)	−	−	−
21.	SS/HD	IV	LN (NSHD)	−	−	−
22.	SS	IV	ERY (+)	−	−	+
			PLQ (+)	−	−	+
			PBL-1992 (+)	−	−	+
			PBL-1993 (+)	−	−	+
			BM (+)	−	−	+
			LN (+LCT)	−	−	+

[a] PP, parapsoriasis; MF, mycosis fungoides; SS, Sezary syndrome; HD, Hodgkins' disease.

[b] National Cancer Institute consensus report staging criteria (Bunn and Lamberg, 1979).

[c] PCH, skin patch; PLQ, skin plaque; TMR, skin tumor; ERY, skin erythroderma; PBL, peripheral blood lymphocytes; LN, lymph node; BM, bone marrow. Year of tissue acquisition is noted when more than one of the same tissue type was analyzed from a given patient.

[d] Status: morphologic and/or molecular evidence of CTCL (+); no evidence of CTCL (−). LCT, histologic evidence of large cell transformation. NSHD, histologic evidence of nodular sclerosing Hodgkin's disease.

[e] −, no PCR amplification products identified. +, PCR amplification products identified.

Sequence Analysis Positive PCR results were confirmed by sequencing. Samples were re-amplified without using the dUTP substitution kit to facilitate subcloning and DNA sequencing. All of the PCR without dUTP, subcloning, and sequencing experiments were performed in a separate laboratory in the Molecular Biology Core Laboratories on the third floor of the Medical Research Building of the Philadelphia Veterans Affairs Medical Center. Following the manufacturer's instructions, PCR amplification products were ligated to TA vectors from an Invitrogen TA cloning kit (Invitrogen, San Diego, CA). DNA minipreparations were performed on cultured colonies, and double-stranded plasmid DNA (3 μg) was denatured with 1 M NaOH/1 mM ethylenediamine tetraacetic acid. After neutralization with 3 M ammonium acetate (pH 5.4), DNA was precipitated with 100% cold ethanol, washed with 70% (vol/vol) ethanol, dried, and annealed immediately with a sequencing primer (M13 reverse or forward primers, Stratagene, La Jolla, CA). DNA sequencing was done by the dideoxynucleotide chain termination method (Sanger *et al*, 1977) using a Sequenase kit (United States Biochemical, Cleveland, OH).

RESULTS

No HTLV-I/II Sequences Detected in Cell Lines We first examined cell lines established from the peripheral blood of seven patients with Sezary syndrome and three healthy adult controls. Molecular and immunophenotypic analysis was performed on each cell line. Southern blot analysis of cell line genomic DNA revealed rearrangements in the immunologic heavy chain locus with a germline configuration in the T-cell receptor β-gene locus and monoclonal integration of EBV (data not shown). Immunohistochemical analysis of cytospin preparations from the cell lines revealed positive CD-19 (B cell) staining whereas CD-2 (T cell) staining was negative (data not shown). Thus, our cell lines consist of monoclonal EBV-transformed B cells. These cell lines are similar to those described in the initial studies reporting the detection of HTLV-I or HTLV-I–like provirus sequences in CTCL (Manzari *et al*, 1987; Hall *et al*, 1991; Zucker-Franklin *et al*, 1991). Southern blot analysis utilizing a full length HTLV-I probe failed to detect genomic integration of HTLV-I proviral DNA in the cell lines (data not shown). PCR amplification of genomic DNA with primers for the HTLV-I *tax* region and *pol* region (identical to previous studies (Hall *et al*, 1991; Zucker-Franklin *et al*, 1991, 1992) failed to detect proviral sequences in the cell lines tested **(Fig 1)**.

No HTLV-I Sequences Detected in Tissue Samples We next analyzed a variety of tissue samples (cutaneous and extracutaneous) from a wide range of disease stages. We sought to determine whether the ability to detect HTLV-I proviral sequences by PCR was related to disease progression or tissue site. Our analysis included 24 skin biopsies (four parapsoriasis, six patch, six plaque, seven tumor, one erythroderma), ten peripheral blood samples, six lymph node biopsies, and three bone marrow biopsies. The results are summarized in **Table I**. All samples were first PCR-amplified for β-actin to ensure DNA integrity, as was done for the cell lines **(Fig 1*F*)**. In skin samples in which the lymphocytic infiltrate was sparse (i.e., patch; see **Table I**), primers for T-cell receptor–γ gene rearrangements were utilized to demonstrate the detection of T-cell infiltrates (Wood *et al*, 1994). In all samples tested, we were able to detect the T-cell infiltrate by PCR (data not shown). Except for positive controls, no sequences of the HTLV-I *tax* region were amplified or detected by Southern blot analysis from the samples. Positive control amplification signals from MT-2 cell line were consistent throughout experimentation **(Fig 1*A,B*)**. As with the analysis of the cell lines above, no PCR amplifications were performed without the use of dUTPs and uracil DNA glycoylase digestion prior to amplification.

PCR amplification with primers for HTLV-I/II *pol* sequences often resulted in variable intensity bands in tissue samples as well as normal controls. When these products were probed with a nested probe specific for HTLV-I *pol*, they were uniformly negative (similar to the cell lines in **Fig 1*C,D***); hence, we did not detect HTLV-I *pol* sequences in the samples tested.

HTLV-II Sequences Detected in One Patient When the products were probed with a nested probe specific for HTLV-II *pol*, they were negative (similar to the cell lines in **Fig 1*E***) with the exception of samples from one patient with advanced CTCL **(Fig 2*C,D*)**. To further characterize this patient, we used primers and nested probes for both HTLV-I and HTLV-II *tax* region and detected the presence of HTLV-II *tax* sequences but not HTLV-I *tax* sequences **(Fig 2*A,B*)**.

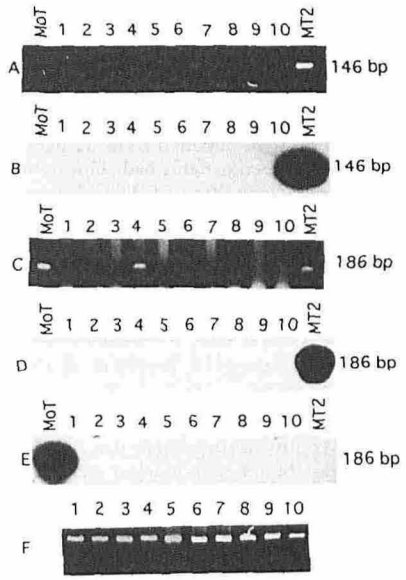

Figure 1. PCR/Southern blot analysis fails to demonstrate HTLV-I *tax* and HTLV-I/II *pol* gene sequences in cell lines. MoT cell line (positive control for HTLV-II), CTCL cell lines (*lanes 1–7*), normal control cell lines (*lanes 8–10*), and MT2 cell line (positive control for *HTLV-I*) were analyzed. *(A)* HTLV-I *tax* PCR products (pX-5' and pX-3' primers). *(B)* PCR products in *(A)* are hybridized with *tax* nested probe (pX-N). *(C)* HTLV-I/II *pol* PCR products (SK110 and SK111 primers). *(D)* PCR products in *(C)* are hybridized with HTLV-I *pol* nested probe (SK112). *(E)* PCR products in *(C)* are hybridized with HTLV-II *pol* nested probe (SK188). *(F)* β-actin gene PCR products. Molecular sizes in base pairs (bp) are indicated on the *right side*.

HTLV-II Amplification Products Contain Endogenous Retroviral Sequences To confirm the identity of the HTLV-II PCR products detected by PCR/Southern blotting, we subcloned them and performed sequence analysis. Interestingly, only one third of the *pol* clones hybridized with the HTLV-II *pol* probe (data not shown). Sequence analysis demonstrated that, whereas some clones contained DNA sequences with perfect homology to the HTLV-II *pol* gene, the majority were products of a human endogenous sequence homologous to the HTLV *pol* gene (Fujihara *et al*, 1994) **(Fig 3)**. It appears that the presence of human endogenous retroviruses is responsible for the nonspecific amplification products that were generated in our HTLV *pol* PCR reactions **(Fig 1C)**. In contrast, all the *tax* clones hybridized to the HTLV-II tax probe. DNA sequence analysis of the clones showed that they are 100% homologous to the HTLV-II tax gene (data not shown). Despite the fact that we were able to detect HTLV-II *tax* and *pol* sequences by PCR, we were unable to document HTLV-II proviral integration in the genome of malignant T cells in the peripheral blood **(Fig 2*E*)**.

DISCUSSION

Since the first molecular report suggesting that a HTLV-I-like retrovirus is associated with CTCL (Manzari *et al*, 1987), an increasing number of reports have been published reporting a variable percentage of detection rates of HTLV-I in patients with CTCL. Despite the fact that no definitive model of HTLV-I infection in CTCL has been advanced or confirmed, some reports have already proposed the use of anti-viral therapies for CTCL

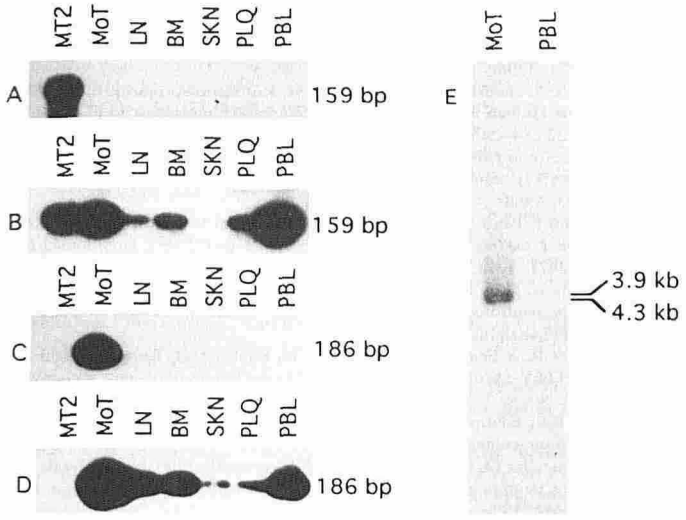

Figure 2. PCR/Southern blot detection of HTLV-II sequences in one patient. MT2 cell line (positive control for HTLV-I), MoT cell line (positive control for HTLV-II), and biopsies of lymph node (LN), bone marrow (BM), erythrodermic skin (SKN), cutaneous plaque (PLQ), and peripheral blood lymphocytes (PBL) were analyzed. HTLV-I/II *tax* PCR products (SK43 and SK44 primers) were hybridized with *(A)* HTLV-I *tax* nested probe (pX-N) and *(B)* HTLV-II *tax* nested probe (SK45). HTLV-I/II *pol* PCR products (SK110 and SK111) were hybridized with *(C)* HTLV-I *pol* nested probe (SK112) and *(D)* HTLV-II *pol* nested probe (SK188). *(E)* Southern blot analysis of *Eco*RI digested genomic DNA from MoT cell line and patient PBL hybridized with a full length HTLV-II probe. Molecular sizes in base pairs (bp) or kilobases (kb) are indicated on the *right side* of each blot.

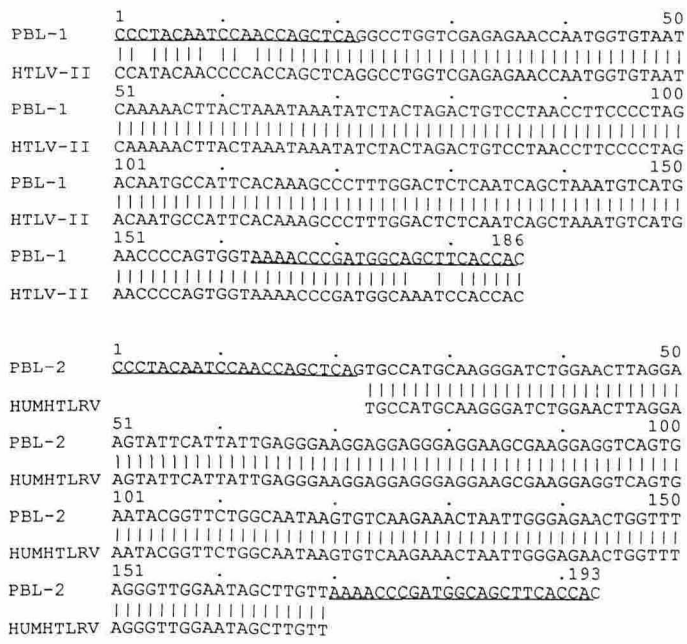

Figure 3. Sequence analysis of HTLV-II *pol* PCR amplification products from patient in Fig 2 demonstrates endogenous retroviral DNA sequences. *Top*, One third of the HTLV-II *pol* gene PCR amplification products (PBL-1) exhibit 100% sequence homology with HTLV-II *pol* gene (HTLV-II) (Genbank accession no. L06854) except the primer regions (*underlined*). *Bottom*, Two thirds of the HTLV-II *pol* gene PCR amplification products (PBL-2) show perfect sequence homology to a human endogenous HTLV-I pseudo-*pol* gene (HUMHTLRV) (Fujihara *et al*, 1994; Genbank accession no. L19438) (primer regions are *underlined*).

(Zucker-Franklin *et al*, 1991) and the use of molecular detection of HTLV-I as a diagnostic aid (Khan *et al*, 1996). With the goal of determining when during disease progression and in what sites could HTLV-I proviral sequences be detected, we sought to develop a model that would be consistent with the natural history and epidemiology of CTCL.

The hallmark of ATL is its occurrence rate in areas in which HTLV-I infection is endemic (Hollsberg and Hafler, 1993). The malignant T cells are predominantly CD4+ and strongly CD25+ (Waldman *et al*, 1984); in up to 70% of cases there is cutaneous involvement that can clinically mimic CTCL (Takatsuki *et al*, 1985). Detection of serum antibodies to HTLV-I surface proteins and the presence of HTLV-I proviral DNA in the malignant T-cell clone are the *sine qua non* of ATL (Takatsuki *et al*, 1985; Hollsberg and Hafler, 1993). In contrast, CTCL is a relatively indolent disorder, and the malignant cells of this disease are CD4+ and weakly CD25+ (Waldman *et al*, 1984). In addition, CTCL occurs sporadically and does not demonstrate case clustering or endemic areas (Weinstock and Horm, 1988). This is in direct contrast with the clear epidemiology of ATL.

Despite establishing cell cultures comparable to previous studies from which HTLV-I detection had been reported (Manzari *et al*, 1987; Hall *et al*, 1991; Zucker-Franklin *et al*, 1991), we were unable to detect HTLV-I proviral DNA sequences. To address the possibilities that viral detection in CTCL may be a function of viral load varying throughout disease progression or early clearance of virus from the skin, we evaluated skin and extracutaneous tissues from all stages of CTCL: from "precursor states" of parapsoriasis to advanced cases with large cell transformation (Salhany *et al*, 1988) and even one patient with concurrent CTCL and Hodgkin's disease. In no instances did we detect HTLV-I retrovirus. Our findings corroborate and extend the studies that found no HTLV-I proviral sequences in skin (Lisby *et al*, 1992; Bazarbachi *et al*, 1993; Boni *et al*, 1996), blood (Capesius *et al*, 1991; Bazarbachi *et al*, 1993), and CTCL-associated lymphoproliferative diseases (Wood *et al*, 1996a, 1996b).

The validity of a model of a casual link of a retrovirus or pathogen with a human disease has always come from independent confirmatory reports subsequent to an initial observation (Winkelman, 1993). The failure to detect *tax* gene proviral sequences, the most conserved HTLV-I region (Hall *et al*, 1991; Korber *et al*, 1991), in our study and the increasing number of other reports (Capesius *et al*, 1991; Lisby *et al*, 1992; Bazarbachi *et al*, 1993; Boni *et al*, 1996) indicate that this has not been the case with retroviral infection and CTCL. In other studies reporting detection of HTLV-I proviral sequences in CTCL, truncated proviruses and low copy number (Hall *et al*, 1991; Zucker-Franklin *et al*, 1991) have been advanced as factors influencing detection rates and the lack of antibody response in CTCL (Lange-Wantzin *et al*, 1986; Srivastava *et al*, 1992). If such mechanisms are associated with HTLV-I infection in CTCL, an entirely new and yet to be confirmed HTLV-I pathogenesis must be operative in CTCL, because it is neither endemic for areas of HTLV-I infection nor does CTCL have the clinicopathologic features of endemic HTLV-I ATL in the majority of cases.

In samples from one patient with advanced CTCL with evidence of histologic large cell transformation, HTLV-II *pol* and *tax* gene sequences were detected. We failed to detect, however, the HTLV-II proviral genomic integration in peripheral blood lymphocytes of this patient by genomic Southern blot analysis. HTLV-II has been isolated from patients with hairy-cell leukemia and chronic neurodegenerative disease (Kalyanaraman *et al*, 1982; Hjelle *et al*, 1992; Jacobson *et al*, 1993); however, its association with these or other diseases has not been definitively defined. The detection of HTLV-II proviral sequences in this patient and a minority of other CTCL patients (Zucker-Franklin *et al*, 1992) can be correlated neither to HTLV-II infection nor to a significant etiologic role in the majority of cases of CTCL.

The reported detection of HTLV-I/II sequences in CTCL patients may represent an effect rather than a cause of the disease. In advanced CTCL, the degree of host immunosuppression is profound, mimicking that of acquired immunodeficiency syndrome (Heald *et al*, 1994). Detection of HTLV-II genes in this study and HTLV-I detection and expression in other reports may be an effect of CTCL-related immunosuppression. Other interpretations are possible for the conflicting data reported in CTCL. Human endogenous retroviral-related sequences comprise up to 0.1% of the human genome (Krieg *et al*, 1992). Some of these have been isolated and proved to be related to HTLV (Mager and Freeman, 1987). The precise function of these genes, if any, has not been defined. The presence of these sequences was confirmed in this study. The previous positive results in other studies may be a result of the amplification of these endogenous genes. Last, not all studies have carefully controlled for contamination and carry-over; unfortunately, this may be responsible for some of the positive results reported in the literature. Wood *et al* have demonstrated "false positive" PCR detection of HTLV-I DNA sequences in CTCL samples resulting from trace contamination (Wood *et al*, 1996a). We are confident that in our study, contamination has been tightly controlled from the onset.

Based on our results and the currently available molecular and epidemiologic data, HTLV-I/II is not a primary etiologic agent in the pathogenesis of CTCL. The association of CTCL with a putative HTLV-like retrovirus, with a currently undefined molecular pathogenesis and epidemiology, has not been excluded. The use of HTLV-I/II–specific reagents, however, has a limited application in the detection and isolation of a new or related retrovirus. Future approaches would benefit from more powerful molecular techniques capable of detecting small differences between two DNA populations (Lisityn *et al*, 1993; Chang *et al*, 1994) and experimental designs emphasizing the biologic behavior and epidemiology of CTCL (Lessin *et al*, 1994).

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REFERENCES

Anagnostopoulos I, Hummel M, Kaudewitz P, Herbst H, Braun-Falco O, Stein H: Detection of HTLV-I proviral sequences in CD30-positive large cell cutaneous T-cell lymphomas. *Am J Pathol* 137:1317–1322, 1990

Bazarbachi A, Saal F, Laroche L, Flageul B, Peries J, de The H: HTLV-I provirus and mycosis fungoides. *Science* 259:1470–1471, 1993

Boni R, Davis-Daneshfar A, Burg G, Fuchs D, Wood G: No detection of HTLV-I proviral DNA in lesional skin biopsies from Swiss and German patients with cutaneous T-cell lymphoma. *Br J Dermatol* 136:282–284, 1996

Bourguin A, Tung R, Galili N, Sklar J: Rapid, nonradioactive detection of clonal T-cell receptor gene rearrangements in lymphoid neoplasms. *Proc Natl Acad Sci USA* 87:8536–8540, 1990

Bunn P, Lamberg S: Report of the committee on staging and classification of cutaneous T-cell lymphomas. *Cancer Treat Reports* 63:725–728, 1979

Capesius C, Saal F, Maero E, Bazarbachi A, Lasneret J, Laroche L, Gessain A, Hojman F, Peries J: No evidence for HTLV-I infection in 24 cases of French and Portuguese mycosis fungoides and Sezary syndrome (as seen in France). *Leukemia* 5:416–419, 1991

Chan W, Hooper C, Wichert R, Benson J, Vardiman J, Hinrichs S, Weisenburger D: HTLV-I sequences in lymphoproliferative disorders. *Diagn Mol Pathol* 2:192–199, 1993

Chang Y, Cesarman E, Pessin M, Lee F, Culpepper J, Knowles D, Moore P: Identification of Herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266:1865–1869, 1994

Detmar M, Pauli G, Anagnostopoulos I, Wunderlich U, Herbst H, Garbe C, Stein H, Orfanos CE: A case of classical mycosis fungoides associated with human T-cell lymphotropic virus type I. *Br J Dermatol* 124:198–202, 1991

D'Incan M, Southteyrand P, Bignon Y, Dastugue B, Claudy A, Desgranges C: Retroviral sequences related to HTLV-I endemic areas. *Eur J Dermatol* 2:363–371, 1992

Edelson RL: Cutaneous T-cell lymphoma: mycosis fungoides, Sezary syndrome and other variants. *J Am Acad Dermatol* 2:89–106, 1980

Finn DT, Jaworsky C, Chooback L, Jensen PJ, Lessin SR: Correlation between clonotypic T-cell receptor beta chain variable region gene expression and aberrant T-cell antigen expression in cutaneous T-cell lymphoma. *J Cutan Pathol*, 23:306–311, 1996

Fujihara K, Du T-L, Selkirk S, Ward P, Rickert M, Hohmann P, Bisaccia E, Greenberg SJ: A New Human Endogenous DNA Sequence Homologous to HTLV-I pol. *Genomics* 22:244–246, 1994

Gauwerky CE, Hoxie J, Nowell PC, Croce CM: Pre-B-cell leukemia with a t(8;14) and a t(14;18) translocation is preceded by follicular lymphoma. *Oncogene* 2:431–435, 1988

Ghosh S, Abrams J, Terunuma H, Vonderheid E, DeFreitas E: Human T-cell leukemia virus type I *tax/rex* DNA and RNA in cutaneous T-cell lymphoma. *Blood* 84:2663–2671, 1994

Greer J, Salhany K, King L. (1993): Cutaneous T cell lymphoma: mycosis fungoides and Sezary syndrome. In: Lee G, *et al* (eds.) *Clinical Hematology*. 9 ed. Lea & Febiger, Philadelphia, pp 2143–2169

Hall WW, Liu CR, Schneewind O, Takahashi H, Kaplan MH, Roupe G, Vahlne A: Deleted HTLV-I provirus in blood and cutaneous lesions of patients with mycosis fungoides. *Science* 253:317–320, 1991

Heald P, Yan S-L, Edelson R: Profound deficiency in normal circulating T cells in erythrodermic cutaneous T-cell lymphoma. *Arch Dermatol* 130:198–203, 1994

Hjelle B, Appenzeller O, Mills R, Alexander S, Torrez-Martinez N, Jahnke R, Goss G: Chronic neurodegenerative disease associated with HTLV-II infection. *Lancet* 339:645–646, 1992

Hollsberg P, Hafler DA: Pathogenesis of diseases induced by human lymphotropic virus type I infection. *N Engl J Med* 328:1173–1182, 1993

Hudson L, Hayes FC: *Practical Immunology*. Blackwell Scientific Publications, London, 1989

Jacobson S, lehky T, Nishimura M, Robinson S, McFarlin DE, Dhibjalbut S: Isolation of HTLV-II from a patient with chronic progressive neurological disease clinically indistinguishable from HTLV-I associated myelopathy/tropical spastic paraparesis. *Ann Neurol* 33:392–396, 1993

Kalyanaraman VS, Sarangadharan MG, Miyoshi I, Blayney D, Golde D, Gallo RC: A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* 218:571–573, 1982

Khan Z, Sebenik M, Zucker-Franklin D: Localization of HTLV-I tax proviral sequences in skin biopsies of patients with mycosis fungoides by *in situ* polymerase chain reaction. *J Invest Dermatol* 106:667–672, 1996

Korber B, Okayama A, Donnelly R, Tachibana N, Essex M: Polymerase chain reaction analysis of defective human T-cell leukemia virus type I proviral genomes in leukemic cells of patients with adult T-cell leukemia. *J Virol* 65:5471–5476, 1991

Krieg AM, Gouriey MF, Perl A: Endogenous retroviruses: Potential etiological agents in autoimmunity. *FASEB J* 6:2537–2544, 1992

Kwok S, Kellogg D, Ehrlich G, Poiesz B, Bhagavati S, Sninsky, JJ: Characterization of a sequence of human T cell leukemia virus type-I from a patient with chronic progressive myelopathy. *J Infect Dis* 158:1193–1197, 1988

Lange-Wantzin G, Thomsen K, Nissen NI, Saxinger D, Gallo RC: Occurrence of human T-cell lymphotropic virus (Type-I) antibodies in cutaneous T-cell lymphoma. *J Am Acad Dermatol* 15:598–602, 1986

Lessin SR, Huebner K, Isobe M, Croce CM, Steinert P: Chromosomal mapping of human keratin genes: Evidence of non-linkage. *J Invest Dermatol* 91:572–578, 1988

Lessin SR, Rook AH, Rovera G: Molecular diagnosis of cutaneous T-cell lymphoma: Polymerase chain amplification of T-cell antigen receptor β-gene rearrangements. *J Invest Dermatol* 96:299–302, 1991

Lessin SR, Vowels BR, Rook AH: Retroviruses and cutaneous T-cell lymphoma. *Dermatol Clin* 12:243–253, 1994

Lisby G, Reitz MS, Vejlsgaard GL: No detection of HTLV-I DNA in punch skin biopsies from patients with cutaneous T-cell lymphoma by the polymerase chain reaction. *J Invest Dermatol* 98:417–420, 1992

Lisityn N, Lisityn N, Wigler M: Cloning the differences between two complex genomes. *Science* 259:946–951, 1993

Lutzner MH, Hobbs JW, Horvath J: Ultrastructure of abnormal cells in Sezary syndrome, mycosis fungoides and parapsoriasis en plaque. *Arch Dermatol* 103:375–386, 1971

MacKie RM: Initial event in mycosis fungoides of the skin is a viral infection of epidermal Langerhans cells. *Lancet* 2:283–285, 1981

Mager DL, Freeman JD: Human endogenous retroviruslike genome with type C pol sequences and gag sequences related to human T-cell lymphotropic viruses. *J Virol* 61:4060–4066, 1987

Manca N, Piacentini E, Gelmi M, Calzavara P, Manganoni MA, Glukhov A, Gargiulo F, Francesco MD, Pirali F, Panfilis GD, Turano A: Persistence of human T cell lymphotropic virus type 1 (HTLV-1) sequences in peripheral blood mononuclear cells from patients with mycosis fungoides. *J Exp Med* 180:1973–1978, 1994

Manzari V, Gismondi A, Barillari G, Morrone S, Modesti A, Albonici L, DeMarchis L, Fazio V, Gradilone A, Zani M, Frati L, Santoni A: HTLV-V: a new human retrovirus isolated in Tac-negative T-cell lymphoma/leukemia. *Science* 238:1581–1583, 1987

Murphy GF: Cutaneous T-cell lymphoma. *Adv Pathol* 1:131–156, 1988

Pancake BA, Zuker-Franklin D, Coutavas EE: The cutaneous T cell lymphoma, mycosis fungoides, is a human T cell lymphotropic virus-associated disease. *J Clin Invest* 95:547–554, 1995

Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: Detection and isolation of type C retrovirus particles from fresh cultured lymphocytes of a patient with cutaneous T cell lymphoma. *Proc Natl Acad Sci USA* 77:7415–7419, 1980

Reddy PE, Mettus RV, DeFreitas E, Wroblewska Z, Cisco M, Koprowski H: Molecular cloning of human T-cell lymphotrophic virus type I-like proviral genome from the peripheral lymphocyte DNA of a patient with chronic neurologic disorders. *Proc Natl Acad Sci USA* 85:3599–3603, 1988

Russo G, Isobe M, Pegoraro L, Finan J, Nowell PC, Croce CM: Molecular analysis of a t(7;14)(q35;q32) chromosome translocation in a T cell leukemia of a patient with ataxia telangectasia. *Cell* 53:137–144, 1988

Salhany KE, Cousar JB, Greer JP, Casey TT, Fields JP, Collins RD: Transformation of cutaneous T cell lymphoma to large cell lymphoma: A clinicopathologic and immunologic study. *Am J Pathol* 132:265–277, 1988

Sanger F, Nicklens S, Coulson AR: DNA sequencing with chain termination inhibitors. *Proc Natl Acad Sci USA* 74:5463- 5467, 1977

Srivastava SBI, Banki K, Perl A: Human T-cell leukemia virus type I or a related retrovirus in patients with mycosis fungoides/Sezary syndrome and Kaposi's sarcoma. *Cancer Res* 52:4391–4395, 1992

Takatsuki K, Yamaguchi K, Kawano F, Hattori T, Nishimura H, Tsuda H, Sanada I, Nakada K, Itai Y: Clinical diversity in adult T-cell leukemia-lymphoma. *Cancer Res* 45:4644S-4645S, 1985

Vowels BJ, Lessin SR, Cassin M, Jaworsky C, Benoit BM, Wolfe JT, Rook AH: Th2 cytokine mRNA expression in peripheral blood and skin in cutaneous T-cell lymphoma. *J Invest Dermatol* 103:669–673, 1994

Waldman TA, Greene WC, Warner CG, Sarin PS, Saxinger C, Blayney DW, William AB, Goldman CK, Bongiovanni K, Sharrow S, Depper JW, Leonard W, Uchiyama T, Gallo RC: Functional and phenotypic comparison of human T-cell leukemia/lymphoma virus positive adult T-cell leukemia with human T-cell leukemia/lymphoma virus negative Sezary leukemia, and their distinction using anti-Tac monclonal antibody identifying the human receptor for T-cell growth factor. *J Clin Invest* 73:1711–1718, 1984

Weinstock MA, Horm JW: Mycosis fungoides in the United States. Increasing incidence and descriptive epidemiolgy. *JAMA* 260:306–308, 1988

Whittaker SJ, Luzzatto L: HTLV-I provirus and mycosis fungoides. *Science* 259:1470, 1993

Winkelman J: HTLV-1 and multiple sclerosis: The link is missing *J Lab Clin Med* 122:230–231, 1993

Wood G, Salvekar A, Schaffer J, Crooks C, Henghold W, Fivenson D, Kim Y-H, Smoller B: Evidence against a role for HTLV-I in the pathogenesis of American cutaneous T-cell lymphoma. *J Invest Dermatol* 107:301–307, 1996a

Wood G, Schaffer J, Boni R, Dummer R, Burg G, Takeshita M, Kikuchi M: No evidence of HTLV-I proviral integration in lymphoproliferative disorders associated with cutaneous T-cell lymphoma. *Am J Pathol* in press, 1996b

Wood G, Tung R, Haeffner A, Crooks C, Liao S, Orozco R, Veelken H, Kadin M, Koh H, Heald P, Barnhill R, Sklar J: Detection of clonal T-cell receptor γ gene rearrangements in early mycosis fungoides/Sezary syndrome by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR/DGGE). *J Invest Dermatol* 103:34–41, 1994

Zucker-Franklin D, Coutavas EE, Rush MG, Zouzias DC: Detection of human T-lymphotropic virus-like particles in cultures of peripheral blood lymphocytes of patients with mycosis fungoides. *Proc Natl Acad Sci USA* 88:7630–7634, 1991

Zucker-Franklin D, Hooper WC, Evatt BL: Human lymphotropic retroviruses associated with mycosis fungoides: evidence that human T-cell lymphotropic-virus Type II (HTLV-II) as well as HTLV-I may play a role in the disease. *Blood* 80:1537–1545, 1992